Fish and Shellfish Immunology 99 (2020) 435–441

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

NK-lysin is highly conserved in European sea bass and gilthead seabream but differentially modulated during the immune response

Yulema Valero[a,b,c], Elena Chaves-Pozo[c], Alberto Cuesta[a,*]

[a] Fish Innate Immune System Group, Department of Cell Biology and Histology, Faculty of Biology, Campus Regional de Excelencia Internacional "Campus Mare Nostrum", University of Murcia, 30100, Murcia, Spain

[b] Departamento de Microbiología y Parasitología, Instituto de Acuicultura, Universidade de Santiago de Compostela, Campus Vida, Santiago de Compostela, Spain

[c] Oceanographic Centre of Murcia, Spanish Institute of Oceanography (IEO), Carretera de la Azohía s/n, 30860, Puerto de Mazarrón, Murcia, Spain

ARTICLE INFO

Keywords:
NK-Lysin
Cell-mediated cytotoxicity
Antimicrobial peptides (AMPs)
European sea bass
Gilthead seabream
Nodavirus (NNV)
Innate immunity

ABSTRACT

Fish NK-lysin (NKL), an orthologous to human granulysin, exerts a dual role as an antimicrobial peptide (AMP) and as a direct executor of T cytotoxic and natural killer cells during the cell-mediated cytotoxic (CMC) response. Although its best-known function is as AMP against bacteria, recent studies point to a special role of NKL in antiviral responses. Nodavirus (NNV) is a spreading threat in Mediterranean aquaculture. In this study, we have identified and compared the expression pattern of European sea bass and gilthead seabream NKL and evaluated its transcription in different tissues and its regulation in head-kidney leucocyte (HKLs) stimulated *in vitro* with different immunostimulants, under CMC response and upon an *in vivo* infection with NNV. Our results showed that *nkl* transcription is highly expressed in spleen, thymus and skin with species-specific differences. Interestingly, the expression pattern in both species was very different upon treatment. While sea bass *nkl* transcription was increased in HKLs by the T mitogen phytohemagglutinin all the stimulators inhibited it in seabream HKLs. Similar results occurred in NNV-infected fish where the transcription was increased in sea bass tissues and down-regulated in seabream. Curiously, during CMC assays, *nkl* transcription was significantly increased in seabream HKLs against NNV-infected fish cell lines but this was not observed in sea bass leucocytes. The potential role of NKL as CMC effector molecule or as AMP in fish will be discussed.

1. Introduction

NK-lysin (NKL; orthologous to human granulysin) is a saposin-like protein involved in the sphingolipid catabolism, synthesized by cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells, and stored in cytolytic granules until released upon stimulation, where plays a role in the cell-mediated cytotoxicity (CMC) [1,2]. In most of the fish species analysed, it has been described the gene presence of one NK-lysin variant, encoding for a putative saposin-B (SapB) domain, conformed by 3 α-helix and 6 conserved cysteines, which stablish 3 stable disulphide bonds [3–9]. On the contrary, in channel catfish (*Ictalurus punctatus*) and zebrafish (*Danio rerio*) there are three or four different variants, respectively [10,11]. Unfortunately, very little is known at protein level, being NKL peptide detected in turbot (*Scophthalmus maximus*) erythrocytes [7], Atlantic salmon (*Salmo salar*) skin mucus [12] or European sea bass (*Dicentrarchus labrax*) eggs, larvae and adult tissues (brain, head-kidney, spleen and muscle) [13]. However, very little is known about its function.

Upon infection, NKL transcription is modulated in several tissues and cell types *in vitro* and *in vivo* [4,6,8,9,13–17] pointing to its relevant function in immunity. Far from being a potential direct effector of fish CTLs and NK cells [1,2], an issue that has not been demonstrated in fish yet, fish NKLs are known to act as an antimicrobial peptide (AMP) and exert antimicrobial functions against bacteria [14,16–19], virus [7,16,18,19] and parasites [15]. NKL interacts with microbial membranes, which ends in their complete disruption [15,18,20]. Regarding virus, latest studies have addressed the concrete antiviral mechanisms of action of NKL. By one side, NKL is shown to decrease viral loads in infected fish [18]. Mechanistically, it also inhibits the replication at early stages of the spring viremia of carp virus (SVCV), in a dose dependent manner, affects the capacity of the viral particles to bind to the membrane and disrupts viral fusion to cells [20]. Moreover, NKL protein is detected in autophagosomes of erythrocytes suggesting that the antiviral activity could be autophagy-dependent [7].

* Corresponding author. Department of Cell Biology and Histology, Faculty of Biology, Regional Campus of International Excellence "Campus Mare Nostrum", University of Murcia, 30100, Murcia, Spain.
E-mail address: alcuesta@um.es (A. Cuesta).

https://doi.org/10.1016/j.fsi.2020.02.049
Received 19 December 2019; Received in revised form 13 February 2020; Accepted 18 February 2020
Available online 20 February 2020

Viral nervous necrosis, also recognised as viral encephalopathy and retinopathy, is an increasingly widespread disease provoked by the nervous necrosis virus (NNV; genus *Betanodavirus*) [21]. Currently, it is known to devastate populations of European sea bass at larvae and juvenile stages raising mortality rates up to 100% [22]. Up to date, though gilthead seabream (*Sparus aurata*) had been considered an asymptomatic carrier that did not suffer mortalities, recent studies have isolated reassortant strains of NNV from natural outbreaks in this fish species in Mediterranean hatcheries [23]. European sea bass and gilthead seabream are the most relevant cultured species in Mediterranean aquaculture so the impact of viral diseases in the economy might be devastating. In this sense, the expansion of the knowledge in immune responses could open new doors to prevent these diseases. In this work, we have identified the *nkl* mRNA of gilthead seabream and compared its expression with that of European sea bass under naïve conditions, after *in vitro* treatment with different immunostimulants and upon *in vivo* infection with NNV. Moreover, we studied the *nkl* gene modulation during the CMC response against mock- or NNV-infected cells. In the light of our results, NKL could be a pivotal molecule in the fight against NNV in European sea bass, whilst in gilthead seabream appears to be more relevant throughout the CMC response.

2. Material and methods

2.1. Animals

European sea bass (*Dicentrarchus labrax*) and gilthead seabream (*Sparus aurata*) specimens were bred and kept at the facilities of the Oceanographic Centre of Murcia, Spanish Institute of Oceanography (COM-IEO) in *Mazarrón* (Spain) in 14 m^3 tanks with 16.2 ± 1.6 °C water temperature, flow-through circuit, suitable aeration, filtration systems and natural photoperiod. The environmental parameters, mortality and food intake, were recorded daily. No historical signs of NNV infection are recorded in these installations. Juvenile specimens of seabream and sea bass of 100–200 and 300–500 g body weight (bw) were used for the study in naïve conditions and *in vitro* stimulations (see below). Seabream and sea bass specimens of 125 ± 25 and 305 ± 77 g bw, respectively were transported to the University of Murcia (Spain) aquaria to perform *in vivo* infections with NNV (see below).

The handling of the specimens was always performed in accordance with the Guidelines of the European Union Council (2010/63/UE) and the Bioethical Committees of the IEO (Permit Number 2010/02) and of the University of Murcia (Permit Number A13150104).

2.2. Sampling

All specimens were sacrificed by an overdose of clove oil (40 μL/L), then weighed and completely bled before sampling.

In order to analyse the constitutive expression in naïve conditions, brain, gill, liver, skin, gonad, gut, head-kidney (HK), spleen and thymus fragments from 6 independent fish were removed and immediately frozen in TRIzol® Reagent (Life Technologies) at −80 °C until used for RNA isolation. Head-kidney leucocyte (HKLs) suspensions were obtained as elsewhere [24]. Briefly, tissue fragments of HK were removed under a sterile environment. Cell straining was performed by forcing pieces of the tissue through a 100 μm nylon mesh with Leibovitz's L15-medium (Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco), 2 mM L-glutamine (Gibco), penicillin (100 IU/ml, Gibco), streptomycin (100 μg/ml, Gibco) and 20 mM HEPES (Gibco). Cells were washed twice by centrifugation and adjusted to 10^7 leucocytes/mL. In all cases, leucocyte viability was determined by the trypan blue exclusion test (> 98%).

2.3. Viruses and bacteria

NNV (strain 411/96, genotype RGNNV) was propagated in the SSN-1 cell line as previously described [25]. In brief, cells were inoculated and incubated at 25 °C until the cytopathic effect (CPE) was extensive (cell monolayer completely disintegrated). Supernatants were harvested and centrifuged to eliminate cell debris. Virus stock was titrated in 96-well plates and expressed as the viral dilution infecting 50% of the cell cultures ($TCID_{50}$), following a methodology formerly defined [26].

Pathogenic bacteria *Vibrio anguillarum* R-82 (*Va*) or *Photobacterium damselae* subsp. *piscicida* (*Pd*) were grown as formerly defined [24]. Absorbance at 600 nm was measured and used to know the concentration based on growth curves. Both bacterial cell cultures were washed, heat-killed at 60 °C for 30 min, washed again and adjusted to 10^{10} bacteria/mL.

2.4. Cell-mediated cytotoxicity assays

CMC was developed as elsewhere [27]. In brief, growing cultures of SAF-1 (ECACC 00122301) or SSN-1 (ECACC 96082808) fish cell lines were detached by standard trypsinization, washed, seeded in flat-bottomed 96 well plates (Nunc) and incubated without (mock-infected) or with 10^6 $TCID_{50}$ NNV/mL (NNV-infected) for 24 h, which were used as targets in the CMC assays. Then, cells were washed and 100 μl of freshly isolated HKLs added as effectors (50 HKLs per target cell). Samples were centrifuged (400g, 1 min) and incubated at 25 °C for 4 h. Finally, samples were centrifuged and cell pellets stored in TRIzol® Reagent at −80 °C for later RNA isolation. CMC samples at 0 h of incubation were used as controls. Samples with targets alone or HKLs alone were also used as technique controls. Infection of the cell lines with NNV was confirmed by real-time PCR (qPCR) [27].

2.5. In vitro treatments and in vivo infection

Aliquots of 10^7 HKLs/mL were incubated in flat-bottomed 48-well microtiter plates (Nunc) at 22 °C during 24 h with: culture medium alone (control), 5 μg/ml lipopolysaccharide (LPS; Sigma), 10 μg/mL phytohemagglutinin (PHA; Sigma), 5 μg/mL concanavalin A (ConA; Sigma), 50 μg/mL synthetic unmethylated cytosine-phosphodiester-guanosine oligodeoxynucleotide 1668 (CpG ODN 1668; sequence 5′-TCCATGACGTTCCTGATGCT-3'; Eurogentec), 25 μg/mL polyinosinic:polycytidylic acid (pI:C; Sigma), 10^8 bacteria/mL (*Va* or *Pd*) or 10^6 $TCID_{50}$ NNV/mL. Afterwards, HKLs were washed with 0.01 M phosphate buffer (PBS) and stored in TRIzol® Reagent at −80 °C for later isolation of RNA.

On the other hand, once at the University of Murcia (Spain) facilities, juvenile specimens (n = 50) of both species were randomly divided into two tanks, kept in 450–500 L running seawater (28‰ salinity) aquaria at 25 °C and with a 12 h light: 12 h dark photoperiod and acclimatised for 15 days prior to the infection. The infection was performed by intramuscular injection of 100 μl containing 10^6 $TCID_{50}$/fish of NNV in SSN-1 culture medium, a control group (mock-infected) was injected with 100 μL of SSN-1 culture medium. Fish (n = 5 fish/group and time) were sampled 1, 7 or 15 days upon infection and brain, HK and gonad tissues were removed and immediately frozen in TRIzol® Reagent and stored at −80 °C for later RNA isolation. We have selected these tissues because i) brain is the main target tissue for NNV replication and infection, ii) HK is the main lymphohematopoietic tissue in fish, and iii) gonad is used by NNV to hide, evade the immune response and be spread to the progeny by vertical transmission.

2.6. Bioinformatic analysis

The mRNA sequence of sea bass *nkl* was already identified and published [28] whilst the sequence of seabream *nkl* has been retrieved from an RNA-seq study. The putative proteins were predicted and the

evolutionary history was inferred using the Neighbour-Joining method [29]. Evolutionary analyses were conducted in MEGA7 [30]. Secondary structures were predicted at http://www.cbs.dtu.dk/services/NetSurfP/.

2.7. Analysis of gene expression by real-time PCR

Total RNA was isolated from TRIzol® Reagent frozen samples following the manufacturer's instructions. One μg of total RNA was treated with DNAse I (Promega) to remove genomic DNA and the first strand of cDNA synthesized by reverse transcription using the Superscript IV (Life Technologies) with an oligo-dT12-18 primer (Life Technologies). Real-time PCR was performed with an ABI PRISM 7500 instrument (Applied Biosystems) using SYBR Green PCR Core Reagents (Applied Biosystems). Reaction mixtures were incubated at 95 °C for 10 min, followed by 40 cycles of 15 s at 95 °C, 1 min at 60 °C, and finally 15 s at 95 °C, 1 min at 60 °C and 15 s at 95 °C. For each mRNA, gene expression was corrected by the elongation factor 1 alpha (*ef1a*) expression in each sample and expressed as $2^{-\Delta Ct}$, where ΔCt is determined by subtracting the *ef1a* Ct value from the target Ct [31]. The primers used were designed specifically using the Oligo Perfect software tool (Thermo Fisher Scientific) and are shown in Table 1. Prior the experiment, the specificity of each primer pair was studied using positive and negative samples. A melting curve analysis of the amplified products validated the primer for specificity. Negative controls with no template were always included in the reactions.

2.8. Statistical analysis

All data are presented as mean ± standard error of the mean (SEM). Results corresponding to the *in vitro* treatments and *in vivo* infection were normalized and expressed as fold change of the treated/infected group compared with the control group.

The statistical analysis of the constitutive expression data in each tissue was analysed by one-way ANOVA to establish statistical differences between tissues followed by the multiple comparison test of Tukey. The data regarding *in vitro* treatments and *in vivo* infections were analysed by a Student-t test to denote statistical differences between treated/infected with control groups. Statistical analyses were conducted using SPSS 20 software. Minimum level of significance was fixed in 0.05.

3. Results and discussion

Gilthead seabream *nkl* mRNA (acc. number MN240490) sequence was retrieved from a RNA-seq study performed in our lab. The protein sequence was deduced and contained 147 amino acids. This predicted sequence was compared to the NKL of the other fish species analysed in this study, the European sea bass. Notwithstanding, sea bass and seabream NKLs have 75% residues conserved, increasing this conservation to 91.9% into the conserved SapB domain, as well as the six conserved cysteine residues that characterize this domain (Fig. 1A). The presence of these six residues implies the formation of three intramolecular disulphide bonds, permitting to get an exceptionally stable structure that is heat- and protease-resistant [32]. This suggests that the European sea bass and gilthead seabream NKL exert similar functions, since the secondary structure is predictably conserved as shown in Fig. 1A. In fact, the detection of the European sea bass NKL in spleen homogenates by Western blot suggests the presence of the monomer and dimers [13]. A phylogenetic tree with the putative NKL proteins was generated to establish the phylogenetic relations among the teleost NKLs (Fig. 1B) and revealed that European sea bass and gilthead seabream NKLs were homologous to their counterparts from other teleost fish species. Indeed, the teleost NKL protein forms an exclusive clade opposed to its mammalian counterparts, providing evidences of high bootstrap in the lineage of teleost fish species. Strikingly, and based on zebrafish NKL variants classification [11], all the analysed fish appear in the same clade than the zebrafish NKLc and NKLd suggesting a similar role, but different to the zebrafish NKLa and NKLb variants. Interestingly, two different and separate groups appear in the fish clade suggesting important differences in their functions. However, direct antibacterial and/or antiviral activities have been demonstrated for fish NKLs, either synthetic peptides or fish-purified, in each clade [3,18–20]. Unfortunately, AMP activity has not been demonstrated for the zebrafish NKLs.

In this work, we compare the expression pattern of the *nkl* gene in a range of tissues of juvenile specimens of European sea bass and gilthead seabream (Fig. 2A). Our results showed a similar pattern of expression in both species with slight differences. In mammals, the main cell types that produces NKL are T lymphocytes and NK cells [1,33], and this is also expected in fish since in both teleost species highest *nkl* transcripts were observed in thymus and spleen. However, high expression levels were also observed in gills of sea bass but not of seabream. In other fish species, the highest expression of *nkl* genes was found in spleen, HK and/or gills [4–6,9–11,16,17], however, in most of the species the thymus had not been tested. Interestingly, some *nkl* specific variants have been also detected in intestine or muscle, suggesting a tissue specialization of the different variants of *nkl* genes in teleost [10,11]. The observation of the high expression of *nkl* gene in the skin and gills of seabream, and in the skin of sea bass, together with the fact that the NKL protein is detected in the skin mucus of Atlantic salmon [12], points to a relevant role of NKL in mucosal surfaces of fish. In addition, our data also showed that the lowest *nkl* mRNA levels appeared in gonad and gut of sea bass or gonad and liver of gilthead seabream (Fig. 2A), supporting the important differences between species.

Although the constitutive expression of the *nkl* gene in HK from both species shows quite similar levels (Fig. 2A), the pattern of expression in HKLs upon immune stimulation is completely different between both species (Fig. 2B). As a first approach to the regulation of the *nkl* gene in leucocytes, we treated HKLs with different mitogens, pathogen-associated molecular patterns (PAMs) or pathogens. In European sea bass HKLs, the *nkl* gene expression was barely modulated and only PHA, a well-known T mitogen, was able to up-regulate the expression of *nkl*, whilst *Va* and NNV down-regulated the *nkl* transcription (Fig. 2B). On the other hand, all the treatments down-regulated *nkl* transcription in seabream HKLs, reaching significance only with LPS, PHA, ODN, *Va*

Table 1
Primer sequences used for gene expression analysis.

Fish species	Protein name	Gene name	Accession number	Sequence (5' – 3')	
European sea bass	NK-lysin	*nkl*	KY801205	F	GAAGAAACACCTCGGGGAAT
				R	GCAGGTCCAACATCTCCTTC
	Elongation factor 1 alpha	*ef1a*	FM019753	F	CGTTGGCTTCAACATCAAGA
				R	GAAGTTGTCTGCTCCCTTGG
Gilthead seabream	NK-lysin	*nkl*	MN240490	F	CGCACCTCGGAGAACTGATT
				R	TCCACGTCGCTTCGGTAAAA
	Elongation factor 1 alpha	*ef1a*	AF184170	F	CTTCAACGCTCAGGTCATCAT
				R	GCACAGCGAAACGACCAAGGGGA

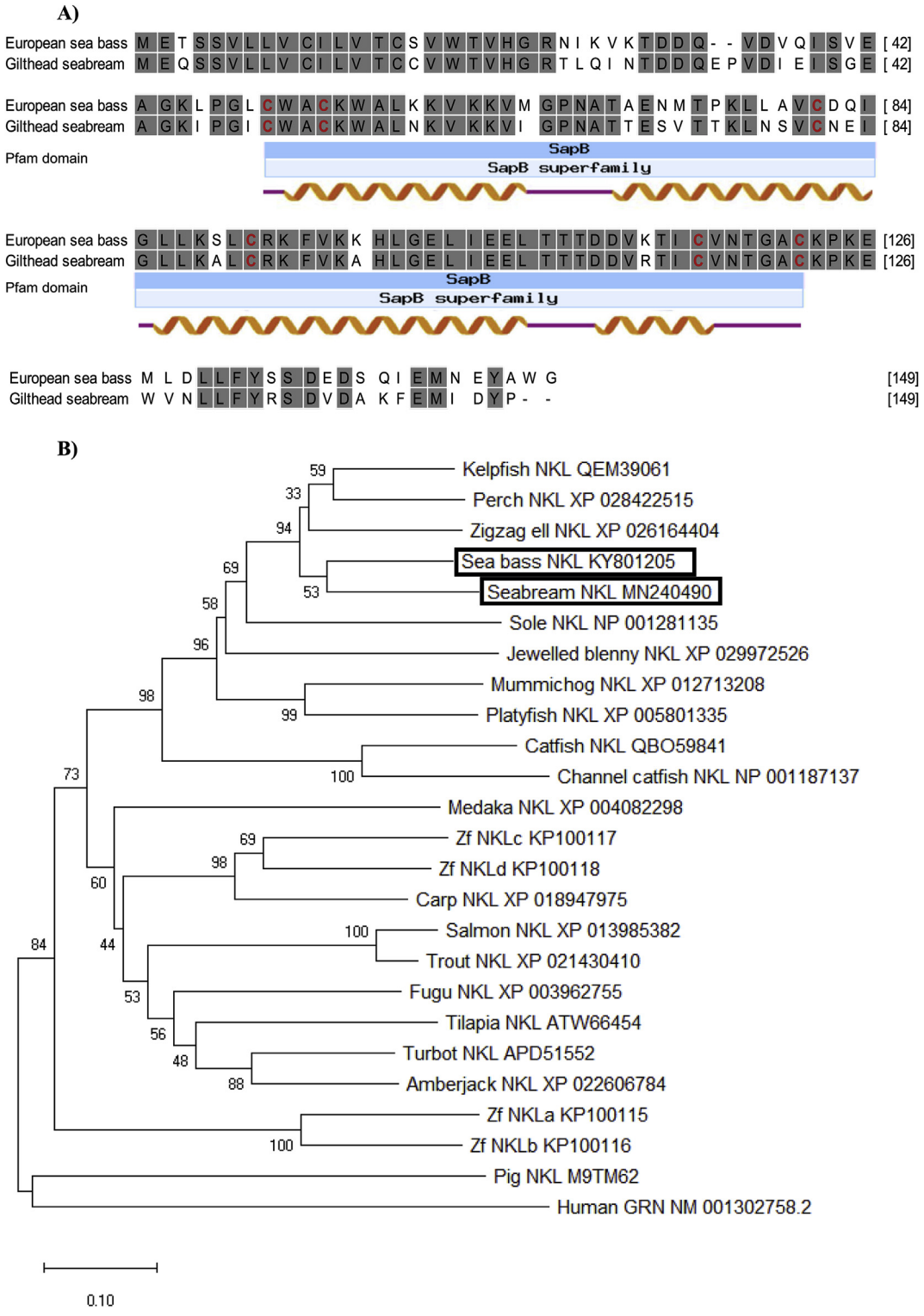

Fig. 1. A) Complete European sea bass and gilthead seabream predicted NK-lysin (NKL) protein sequences (acc. numbers KY801205 and MN240490, respectively) were annealed using ClustalW program. The saposinB (sapB) domain and the prediction of its secondary structure for NKLs of both species were also represented. Conserved cysteine residues are in red. Identity residues are in boxed grey. Helix. Coil. B) Phylogenetic analysis of the putative NK-lysin protein of gilthead seabream and European sea bass with related sequences of fish and mammalian NKL proteins. The phylogenetic tree was drawn following the Neighbour-Joining method for the analysis of evolutionary relationships. Genetic distances were calculated based on protein differences (p-distance) with pairwise deletion. The number at each node indicates the percentage of bootstrapping after 10,000 replications. GenBank accession numbers are shown. Zf, zebrafish. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

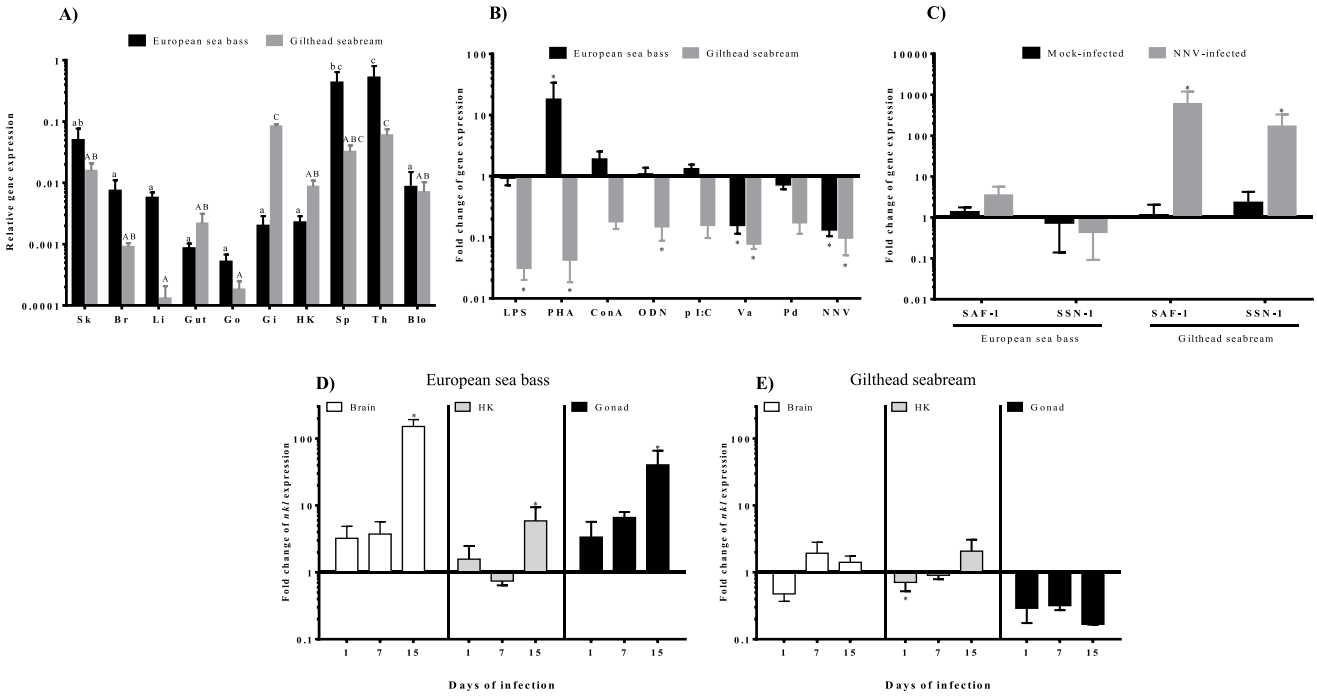

Fig. 2. Pattern of expression of the NK-lysin coding gene (*nkl*) in naïve European sea bass and gilthead seabream tissues (A), in HKLs treated *in vitro* with different stimulators (B), in the cell-mediated cytotoxic against mock- or NNV-infected fish cell lines (C) and in brain, HK or gonad after the *in vivo* infection with NNV (D,E) studied by real-time PCR. In A, data represent the mean relative expression to the endogenous controls *ef1a* gene ± standard error of the mean (SEM) in naïve tissues (n = 3) and letters denote differences between tissues according to ANOVA and Tukey's post-hoc test ($p < 0.05$). In B-E, data represent the mean fold change of the relative gene expression ± SEM in treated/infected groups compared with controls (n = 5–6) and asterisks denote differences among treated/infected and control group according to Student-t test ($p < 0.05$). Sk, skin; Br, brain; Li, liver; Go, gonad; HK, head-kidney; Sp, spleen; Th, thymus; Blo, blood; LPS, lipopolysaccharide; PHA, phytohemagglutinin; ConA, concanavalin A; ODN, synthetic analogue to bacterial DNA; pI:C, synthetic analogue of double-stranded RNA; *Va*, *Vibrio anguillarum*; *Pd*, *Photobacterium damselae*; NNV, nodavirus.

and NNV treatments (Fig. 2B). As far as we are concerned, this is the first study where HKLs are treated to elucidate the modulation of *nkl* gene *in vitro*. Such differences between the two fish species could reside in the cell populations present in the HKLs. For example, seabream HKLs contain a large and very active population of acidophilic granulocytes with phagocytic functions while, in sharp contrast, sea bass possess neutrophils instead, with lower phagocytic capacity [35–37]. Unfortunately, proper quantitative comparisons about B, T, NK, non-specific cytotoxic cells (NCCs) or monocyte-macrophages in both fish species are not available. In this sense, the unique study at this respect describes that myeloid cells from the kidney of zebrafish show the lowest *nkl* transcription followed by lymphoid and precursor cells and that $Rag^{-/-}$ zebrafish show increased transcription of *nklb*, *nklc* and *nkld* variants, and increased *nkla*, when compared to wild-type specimens [11]. Therefore, whether the observed differences in the seabream and sea bass HKLs reside in the differential leucocyte subpopulations, their maturity or functionality is unknown and merits deeper characterization. Similar to the present data, the gene expression of histones *h1* and *h2b*, also considered as AMPs, showed a different species-specific pattern in HKLs. In fact, *in vitro* exposure to most of the mitogens and PAMPs unaffected *h1* and *h2b* transcription in sea bass HKLs while decreased *h1* and up-regulated *h2b* in seabream HKLs [24]. Curiously, the NNV or *Va* exposure down-regulated the transcripts of *nkl* in HKLs of both species, while up-regulated the *h2b* gene expression in gilthead seabream [24]. Additionally, transcription of hepcidin, another important AMP, was always up-regulated with the same *stimuli* in seabream HKLs [34]. Based on these observations further studies would be needed to ascertain the specific role of HKLs in the immune response based on AMPs and why this response is so different between these two fish species.

The first function identified in mammals for NKL was related to the CMC activity. Unfortunately, only one study has related the up-regulation of fish *nkl* gene expression with the NCCs activity after the treatment with attenuated bacteria [6]. For this reason, we also evaluated *nkl* transcription in a CMC assay with different cell lines as targets (Fig. 2C). However, neither seabream nor sea bass HKLs up-regulated the transcription of *nkl* during CMC assays against fish target cells alone. Strikingly, seabream HKLs greatly increased the transcription of the *nkl* gene when incubated with NNV-infected cells, but sea bass HKLs failed to do so (Fig. 2C). These data clearly point to a different role of NKL in both species where CMC might be mediated by NKL in gilthead seabream upon NNV. These data greatly support previous studies where CMC activity was increased in gilthead seabream HKLs but not in European sea bass against NNV-infected cells [27,38] and could be another factor related to the resistance to NNV for seabream and to the susceptibility for sea bass. This is the first study describing a potential role of NKL in fish CMC response.

Interestingly, and in sharp contrast to the down-regulation of the *nkl* gene expression in European sea bass HKLs upon incubation with NNV and the lack of regulation when exposed to NNV-infected target cells, it was up-regulated in HK tissue upon an *in vivo* infection with NNV (Fig. 2D), as also occurred in previous infections performed either intravitreally or intramuscularly [13,28]. However, when we compare the *nkl* expression upon NNV infection of juveniles of European sea bass and gilthead seabream a completely different pattern of transcription was observed (Fig. 2D and E). In European sea bass, *nkl* gene expression was generally increased upon infection but did it to a significant extent at 15 days of infection in brain, HK and gonad (Fig. 2D) while in gilthead seabream it was generally down-regulated, reaching significance at 1 day of infection in HK (Fig. 2E). The behaviour of this *nkl* mRNA level upon NNV is quite similar to other AMPs and immune-related genes, which are greatly enhanced in brain and retina (target tissues) and HK of European sea bass, but unaltered or even down-regulated in gilthead seabream [13,24,27,39–43], probably related to the

differences in susceptibility between fish species. By contrast, *nkl* transcription was not regulated in zebrafish upon pathogenic SVCV [11], while it was associated to the turbot resistance to Rhabdovirus, probably via its involvement in the autophagy process [7]. Our data increase the evidence that there are important differences between fish species immune response and immune molecule function and regulation, leading to the need of further comparative studies. In addition, it was demonstrated that NNV is able to colonize and replicate into the testis of European sea bass and gilthead seabream triggering a latent infection, probably as an evading mechanism to spread to the progeny [39]. At this regard, the great up-regulation of *nkl* gene in the gonad of European sea bass (Fig. 2D) could indicate a role in anti-NNV response since only viral RNA, and not viral proteins, were detected in European sea bass testis while both viral RNA and proteins were found in gilthead seabream testis [39]. Whether the mRNA levels of *nkl* are related to the immune response based on AMP and/or the cytotoxic cells function in both species remains to be elucidated. In European sea bass and gilthead seabream, both the NCC activity and the transcription of its marker *nccrp1*, was increased in HK and brain of both fish species upon NNV infection but with a different time pattern [37]. In addition, the role of NKL peptides is only supported by the fact that synthetic NKL peptides or the recombinant protein show antimicrobial functions *in vitro* [4,8,9,15,18–20,43]. As far as we are concerned, there are no studies boarding the possible different functions of NKL upon a viral infection. However, these considerations altogether highlight the necessity to study the role of NKL peptide as AMP or as CMC executor and deserve further studies.

4. Conclusion

To resume, NKL of gilthead seabream is homologous to other NKLs of teleost fish, shows great conservation in the SapB domain and in the predicted secondary structure, suggesting that the function also might be similar amongst fish species. The *nkl* gene shows similar tissue-distribution in European sea bass and gilthead seabream with high mRNA levels mainly in spleen and thymus and its expression in HK was quite similar between species. However, the *nkl* gene expression is barely altered, induced or decreased in HKLs of European sea bass, but always decreased in gilthead seabream, after *in vitro* stimulation with several mitogens, PAMPs and pathogens (including NNV), suggesting differences in the leucocyte subpopulations, maturity or functionality. In spite of it, gilthead seabream HKLs transcript levels of *nkl* sharply increase during the CMC response against NNV-infected target cells, but not in European sea bass, pointing to its involvement in the CMC response in gilthead seabream. On the contrary, the *in vivo* infection of European sea bass with NNV triggers a great up-regulation of *nkl* gene expression 15 days after infection in brain, HK and gonad, suggesting a potential role of NKL in antiviral response in this species, while in gilthead seabream was unaltered or down-regulated. Nevertheless, the knowledge about NKL functions in teleost fish is still scarce and further studies are needed to enlighten it.

CRediT authorship contribution statement

Yulema Valero: Investigation, Writing - review & editing. **Elena Chaves-Pozo:** Conceptualization, Funding acquisition, Writing - review & editing, Supervision. **Alberto Cuesta:** Conceptualization, Funding acquisition, Writing - review & editing, Supervision.

Acknowledgments

Yulema Valero thanks to Spanish Institute of Oceanography for her PhD grant and *Ministerio de Economía y Competitividad* (MINECO) for her *Juan de la Cierva-Formación*'s research contract. This work was supported by MINECO and FEDER [grants AGL2013-43588-P and AGL2016-74866-C3-1-R], *Fundación Séneca, Grupo de Excelencia de la Región de Murcia* [grant 19883/GERM/15] and Spanish Institute of Oceanography [grant NODAMED].

References

[1] M. Andersson, H. Gunne, B. Agerberth, A. Boman, T. Bergman, B. Olsson, A. Dagerlind, H. Wigzell, H.G. Boman, G.H. Gudmundsson, NK-lysin, structure and function of a novel effector molecule of porcine T and NK cells, Vet. Immunol. Immunopathol. 54 (1996) 123–126, https://doi.org/10.1016/S0165-2427(96)05677-2.

[2] S.V. Peña, A.M. Krensky, Granulysin, a new human cytolytic granule-associated protein with possible involvement in cell-mediated cytotoxicity, Semin. Immunol. 9 (1997) 117–125, https://doi.org/10.1006/smim.1997.0061.

[3] J. Acosta, F. Roa, I. González-Chavarría, A. Astuya, R. Maura, R. Montesino, C. Muñoz, F. Camacho, P. Saavedra, A. Valenzuela, O. Sánchez, J.R. Toledo, *In vitro* immunomodulatory activities of peptides derived from *Salmo salar* NK-lysin and cathelicidin in fish cells, Fish Shellfish Immunol. 88 (2019) 587–594, https://doi.org/10.1016/j.fsi.2019.03.034.

[4] F.F. Ding, C.H. Li, J. Chen, Molecular characterization of the NK-lysin in a teleost fish, *Boleophthalmus pectinirostris*: antimicrobial activity and immunomodulatory activity on monocytes/macrophages, Fish Shellfish Immunol. 92 (2019) 256–264, https://doi.org/10.1016/j.fsi.2019.06.021.

[5] P. Han, G. Tian, W. Gao, H. Yuan, D. Deng, W. Zhang, Q. Xu, Expression analysis of cathelicidin and NK-lysin in Dabry's sturgeon (*Acipenser dabryanus*), J. Appl. Ichthyol. 35 (2019) 825–830, https://doi.org/10.1111/jai.13922.

[6] Y. Huang, Q. Zheng, J. Niu, J. Tang, B. Wang, E.D. Abarike, Y. Lu, J. Cai, J. Jian, NK-lysin from *Oreochromis niloticus* improves antimicrobial defence against bacterial pathogens, Fish Shellfish Immunol. 72 (2018) 259–265, https://doi.org/10.1016/j.fsi.2017.11.002.

[7] P. Pereiro, A. Romero, P. Díaz-Rosales, A. Estepa, A. Figueras, B. Novoa, Nucleated teleost erythrocytes play an NK-lysin- and autophagy-dependent role in antiviral immunity, Front. Immunol. 8 (2017) 1458, https://doi.org/10.3389/fimmu.2017.01458.

[8] G.L. Wang, M.C. Wang, Y.L. Liu, Q. Zhang, C.F. Li, P.T. Liu, E.Z. Li, P. Nie, H.X. Xie, Identification, expression analysis, and antibacterial activity of NK-lysin from common carp *Cyprinus carpio*, Fish Shellfish Immunol. 73 (2018) 11–21, https://doi.org/10.1016/j.fsi.2017.11.030.

[9] Q.J. Zhou, J. Wang, Y. Mao, M. Liu, Y.Q. Su, Q.Z. Ke, J. Chen, W.Q. Zheng, Molecular structure, expression and antibacterial characterization of a novel antimicrobial peptide NK-lysin from the large yellow croaker *Larimichthys crocea*, Aquaculture 500 (2019) 315–321, https://doi.org/10.1016/j.aquaculture.2018.10.012.

[10] Q. Wang, Y. Wang, P. Xu, Z. Liu, NK-lysin of channel catfish: gene triplication, sequence variation, and expression analysis, Mol. Immunol. 43 (2006) 1676–1686, https://doi.org/10.1016/j.molimm.2005.09.023.

[11] P. Pereiro, M. Varela, P. Diaz-Rosales, A. Romero, S. Dios, A. Figueras, B. Novoa, Zebrafish NK-lysins: first insights about their cellular and functional diversification, Dev. Comp. Immunol. 51 (2015) 148–159, https://doi.org/10.1016/j.dci.2015.03.009.

[12] Y. Valero, J. Cortés, L. Mercado, NK-lysin from skin-secreted mucus of Atlantic salmon and its potential role in bacteriostatic activity, Fish Shellfish Immunol. 87 (2019) 410–413, https://doi.org/10.1016/j.fsi.2019.01.034.

[13] Y. Valero, M. Arizcun, J. Cortés, F. Ramírez-Cepeda, F. Guzmán, L. Mercado, M.Á. Esteban, E. Chaves-Pozo, A. Cuesta, NK-lysin, Dicentracin and hepcidin antimicrobial peptides in European sea bass. Ontogenetic development and modulation in juveniles by nodavirus, Dev. Comp. Immunol. 103 (2020) 103516, https://doi.org/10.1016/j.dci.2019.103516.

[14] I. Hirono, H. Kondo, T. Koyama, N.R. Arma, J.Y. Hwang, R. Nozaki, N. Midorikawa, T. Aoki, Characterization of Japanese flounder (*Paralichthys olivaceus*) NK-lysin, an antimicrobial peptide, Fish Shellfish Immunol. 22 (2007) 567–575, https://doi.org/10.1016/j.fsi.2006.08.003.

[15] R. Lama, P. Pereiro, M.M. Costa, J.A. Encinar, R.M. Medina-Gali, L. Pérez, J. Lamas, J. Leiro, A. Figueras, B. Novoa, Turbot (*Scophthalmus maximus*) NK-lysin induces protection against the pathogenic parasite *Philasterides dicentrarchi* via membrane disruption, Fish Shellfish Immunol. 82 (2018) 190–199, https://doi.org/10.1016/j.fsi.2018.08.004.

[16] M. Zhang, H. Long, L. Sun, A NK-lysin from *Cynoglossus semilaevis* enhances antimicrobial defense against bacterial and viral pathogens, Dev. Comp. Immunol. 40 (2013) 258–265, https://doi.org/10.1016/j.dci.2013.03.005.

[17] Q.J. Zhou, J. Wang, M. Liu, Y. Qiao, W.S. Hong, Y.Q. Su, K.H. Han, Q.Z. Ke, W.Q. Zheng, Identification, expression and antibacterial activities of an antimicrobial peptide NK-lysin from a marine fish *Larimichthys crocea*, Fish Shellfish Immunol. 55 (2016) 195–202, https://doi.org/10.1016/j.fsi.2016.05.035.

[18] M. Zhang, M.-f. Li, L. Sun, NKLP27: a teleost NK-lysin peptide that modulates immune response, induces degradation of bacterial DNA, and inhibits bacterial and viral infection, PloS One 9 (2014) e106543, , https://doi.org/10.1371/journal.pone.0106543.

[19] R. León, M. Ruiz, Y. Valero, C. Cárdenas, F. Guzmán, M. Vila, A. Cuesta, Exploring small cationic peptides of different origin as potential antimicrobial agents in aquaculture, Fish Shellfish Immunol. 98 (2020) 720–727, https://doi.org/10.1016/j.fsi.2019.11.019.

[20] A. Falco, R.M. Medina-Gali, J.A. Poveda, M. Bello-Perez, B. Novoa, J.A. Encinar, Antiviral activity of a turbot (*Scophthalmus maximus*) NK-lysin peptide by inhibition of low-pH virus-induced membrane fusion, Mar. Drugs 17 (2019) 20, https://doi.

org/10.3390/md17020087.
[21] OIE, Viral encephalopaty and retinopathy, World Organisation for Animal Health (Ed.), Manual of Diagnostic Test for Aquatic Animals, 2019.
[22] J.Z. Costa, K.D. Thompson, Understanding the interaction between Betanodavirus and its host for the development of prophylactic measures for viral encephalopathy and retinopathy, Fish Shellfish Immunol. 53 (2016) 35–49, https://doi.org/10.1016/j.fsi.2016.03.033.
[23] A. Toffan, F. Pascoli, T. Pretto, V. Panzarin, M. Abbadi, A. Buratin, R. Quartesan, D. Gijon, F. Padros, Viral nervous necrosis in gilthead sea bream (*Sparus aurata*) caused by reassortant betanodavirus RGNNV/SJNNV: an emerging threat for Mediterranean aquaculture, Sci. Rep. 2 (2017) 46755, https://doi.org/10.1038/srep46755.
[24] Y. Valero, M. Arizcun, M.Á. Esteban, A. Cuesta, E. Chaves-Pozo, Transcription of histones H1 and H2B is regulated by several immune stimuli in gilthead seabream and European sea bass, Fish Shellfish Immunol. 57 (2016) 107–115, https://doi.org/10.1016/j.fsi.2016.08.019.
[25] G. Frerichs, H.D. Rodger, Z. Peric, Cell culture isolation of piscine neuropathy nodavirus from juvenile sea bass, *Dicentrarchus labrax*, J. Gen. Virol. 77 (1996) 2067–2071, https://doi.org/10.1099/0022-1317-77-9-2067.
[26] L.J. Reed, H. Müench, A simple method of estimating fifty per cent endpoints, Am. J. Epidemiol. 27 (1938) 493–497.
[27] E. Chaves-Pozo, Y. Valero, A. Esteve-Codina, J. Gómez-Garrido, M. Dabad, T. Alioto, J. Meseguer, M.Á. Esteban, A. Cuesta, Innate cell-mediated cytotoxic activity of European sea bass leucocytes against nodavirus-infected cells: a functional and RNA-seq study, Sci. Rep. 7 (2017) 15396, https://doi.org/10.1038/s41598-017-15629-6.
[28] Y. Valero, B. Boughlala, M. Arizcun, S. Patel, I.U. Fiksdal, M.Á. Esteban, J. De Juan, J. Meseguer, E. Chaves-Pozo, A. Cuesta, Genes related to cell-mediated cytotoxicity and interferon response are induced in the retina of European sea bass upon intravitreal infection with nodavirus, Fish Shellfish Immunol. 74 (2018) 627–636, https://doi.org/10.1016/j.fsi.2018.01.034.
[29] N. Saitou, M. Nei, The neighbor-joining method: a new method for reconstructing phylogenetic trees, Mol. Biol. Evol. 4 (1987) 406–425, https://doi.org/10.1093/oxfordjournals.molbev.a040454.
[30] S. Kumar, G. Stecher, K. Tamura, MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets, Mol. Biol. Evol. 33 (2016) 1870–1874, https://doi.org/10.1093/molbev/msw054.
[31] M.W. Pfaffl, A new mathematical model for relative quantification in real-time RT-PCR, Nucleic Acids Res. 29 (2001) e45.
[32] Y. Kishimoto, M. Hiraiwa, J.S. O'Brien, Saposins: structure, function, distribution, and molecular genetics, J. Lipid Res. 33 (1992) 1255–1267.
[33] S.V. Peña, D.A. Hanson, B.A. Carr, T.J. Goralski, A.M. Krensky, Processing, subcellular localization, and function of 519 (granulysin), a human late T cell activation molecule with homology to small, lytic, granule proteins, J. Immunol. 158 (1997) 2680–2688.
[34] A. Cuesta, J. Meseguer, M.Á. Esteban, The antimicrobial peptide hepcidin exerts an important role in the innate immunity against bacteria in the bony fish gilthead seabream, Mol. Immunol. 45 (2008) 2333–2342, https://doi.org/10.1016/j.molimm.2007.11.007.
[35] M.Á. Esteban, V. Mulero, J. Muñoz, J. Meseguer, Methodological aspects of assessing phagocytosis of *Vibrio anguillarum* by leucocytes of gilthead seabream (*Sparus aurata* L.) by flow cytometry and electron microscopy, Cell Tissue Res. 293 (1998) 133–141, https://doi.org/10.1007/s004410051105.
[36] A. do Vale, A. Afonso, M.T. Silva, The professional phagocytes of sea bass (*Dicentrarchus labrax* L.): cytochemical characterisation of neutrophils and macrophages in the normal and inflamed peritoneal cavity, Fish Shellfish Immunol. 13 (2002) 183–198, https://doi.org/10.1006/fsim.2001.0394.
[37] E. Chaves-Pozo, F.A. Guardiola, J. Meseguer, M.Á. Esteban, A. Cuesta, Nodavirus infection induces a great innate cell-mediated cytotoxic activity in resistant, gilthead seabream, and susceptible, European sea bass, teleost fish, Fish Shellfish Immunol. 33 (2012) 1159–1166, https://doi.org/10.1016/j.fsi.2012.09.002.
[38] E. Chaves-Pozo, Y. Valero, M.T. Lozano, P. Rodríguez-Cerezo, L. Miao, V. Campo, M.Á. Esteban, A. Cuesta, Fish granzyme A shows a greater role than granzyme B in fish innate cell-mediated cytotoxicity, Front. Immunol. 10 (2019) 2579, https://doi.org/10.3389/fimmu.2019.02579.
[39] Y. Valero, M. Arizcun, M.Á. Esteban, I. Bandín, J.G. Olveira, S. Patel, A. Cuesta, E. Chaves-Pozo, Nodavirus colonizes and replicates in the testis of gilthead seabream and European sea bass modulating its immune and reproductive functions, PloS One 10 (2015) e0145131, , https://doi.org/10.1371/journal.pone.0145131.
[40] Y. Valero, A. García-Alcázar, M.Á. Esteban, A. Cuesta, E. Chaves-Pozo, Antimicrobial response is increased in the testis of European sea bass, but not in gilthead seabream, upon nodavirus infection, Fish Shellfish Immunol. 44 (2015) 203–213, https://doi.org/10.1016/j.fsi.2015.02.015.
[41] Y. Valero, P. Morcillo, J. Meseguer, F. Buonocore, M.Á. Esteban, E. Chaves-Pozo, A. Cuesta, Characterization of the interferon pathway in the teleost fish gonad against the vertically transmitted viral nervous necrosis virus, J. Gen. Virol. 96 (2015) 2176–2187, https://doi.org/10.1099/vir.0.000164.
[42] H. Cordero, C.H. Li, E. Chaves-Pozo, M.Á. Esteban, A. Cuesta, Molecular identification and characterization of haptoglobin in teleosts revealed an important role on fish viral infections, Dev. Comp. Immunol. 76 (2017) 189–199, https://doi.org/10.1016/j.dci.2017.06.006.
[43] S. Cai, J. Wang, D. Chen, X. Dong, T. Liu, Y. Zeng, X. Wang, D. Wu, Expression, purification and antibacterial activity of NK-lysin mature peptides from the channel catfish (*Ictalurus punctatus*), Appl. Sci. 6 (2016) 240, https://doi.org/10.3390/app6090240.